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THE UNIVERSITY OF ALBERTA

Purification of Measles Virus Hemagglutinin for Tryptic Analysis by High Performance Liquid Chromatography

by

(C) JAMES A. WILEY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

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Date. February 3rd, 1984

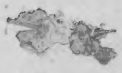
ABSTRACT

The hemagglutinin protein of the Edmonston and Hallé strains of measles virus was purified by a multi-step centrifugation and chromatographic method developed for this study. Treatment of the purified viral material with Triton X-100 released the hemagglutinin protein from the viral membrane. Subsequent purification of the hemagglutinin by ultra-centrifugation and anion-exchange chromatography enabled preservation of the biological of the hemagglutinin, which was an aim of this project.

During development of this purification procedure, SDS-PAGE analysis revealed the presence of a hemagglutinin doublet as well as the usually observed single band of hemagglutinin, in both the Edmonston and Hallé strains. The doublet polypeptides behaved similarly to the single hemagglutinin polypeptide with the exception of being 5,000 to 9,000 less in molecular weight.

The purified hemagglutinin preparations were subsequently proteolytically digested with TPCK-trypsin. The resulting tryptic peptides were analyzed by reversed-phase high performance liquid chromatography. Comparison of the tryptic peptide profiles showed that differences exist in the hemagglutinin of these two strains of measles virus. These differences, seen late in the Hallé profile, indicate the presence of hydrophobic amino acid sequences not contained in the Edmonston hemagglutinin. Although these differences involve relatively small peaks, they may

potentially be of importance.



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ABBREVIATIONS

a.u.	absorbance units
BSA	bovine serum albumin
C	Celsius
CDS	computer data system
cm	centimeter
CNS	Central Nervous System
CPE	Cytopathic Effect
EDTA	Ethylenediamine tetraacetic acid
F	Hemolysin
F_1	Hemolysin subunit 1
F_2	Hemolysin subunit
g	gravity
H	Hemagglutinin
HPLC	High Performance Liquid Chromatography
hrs	hours
IU	International Units
KHz	kilohertz
L	large
M	matrix (according to text)
M	molarity (accordng to text)
mA	milliamperes
MEM	minimal essential media
ml	milliliter
mm	millimeter
mRNA	messenger ribonucleic acid
MVE	measles virus - Edmonston strain

MVH	measles virus - Hallé strain
MW	molecular weight
N	normality
NaCl	sodium chloride
nm	nanometer
NP	nucleoprotein
ODS	octadecylsilane
P	polymerase
PBS	phosphate buffered saline
RNA	ribonucleic acid
S	small
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SSPE	subacute sclerosing panencephalitis
TPCK	L-tosylamide 2-phenyl ethyl chloroethyl ketone
Tris	Tris(Hydroxymethyl)aminoethane
Tris-HCl	Tris-hydrochloride
TG	Tris-glycine
TNE	Tris-sodium EDTA
TX-100	Triton X-100
ul	microliter
UV	ultraviolet

I. Introduction

Acute measles virus infection is one of the most common childhood diseases. Infection by this virus is easily recognized by its characteristic signs, ie. the extensive maculopapular rash covering the body, the accompanying fever and the Koplik's spots seen in the oral cavity.

Variants of measles virus have been found to be responsible for the rare, fatal central nervous system disease, subacute sclerosing panencephalitis (SSPE). The involvement of measles virus in SSPE was first noted in the mid-1960's by Bouteille, Fountaine, Vedrenne and Delarue (1965). Their discovery was quickly confirmed by others (Connolly, Allen, Hurwitz and Millar, 1967; Payne, Joseph, Baublis and Hideo, 1968; Horta-Barbosa, Fuccillo, London, Jabbour, Zeman and Sever, 1969). This disease was found to be a persistent infection initiated during the patient's childhood exposure to some form of measles virus, either through vaccination or natural infection. The age of initial measles virus infection in SSPE patients was usually 2 years of age or less, whereas for those patients who did not develop SSPE, the average age of infection was 4-5 years. The appearance of SSPE signs was generally 6-7 years after the initial measles virus infection (Wechsler and Meissner, 1982).

During the 1970's, research into slow viral diseases of the central nervous system increased in the hope that a better understanding of the relationship between measles and

SSPE could be achieved. It was anticipated that with the knowledge gained of this rare disease, progress could be made towards unravelling some of the questions concerning more common CNS diseases such as multiple sclerosis (Morgan and Rapp, 1977; Norrby, Orvell, Vandvik and Cherry, 1981).

Measles virus is a member of the family Paramyxoviridae, genus Morbillivirus. Each virus of this family is enveloped and has an outer diameter of 100 to 300 nm. The genus Morbillivirus also consists of canine distemper virus, rinderpest virus and peste-des-petits ruminants virus. The genome of these viruses is composed of a negative sense, single strand of RNA having a molecular weight of 4.5×10^4 (Baczko, Billeter and ter Meulen, 1983).

The partially cellular-derived envelope has been the source of a constant problem in attempts to isolate and purify measles virus proteins. Numerous minor cellular components which become associated with the virions frequently appear as bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) preparations. Other paramyxoviruses seem to be relatively free of this problem. Mountcastle and Choppin (1977) have attributed this unique problem to an inefficient budding process in which host cell membrane and cytoplasmic components are retained rather than excluded from the maturing virion. The resulting virions are pleomorphic in shape, resembling microvilli-like bags, and contain viral as well as host cell material (Mountcastle and Choppin, 1977).

Another factor contributing to this purification problem is the known inability of measles virus to shut off host cell protein synthesis during infection (Rima, 1983; Bellini, Silver and McFarlin, 1983).

SDS-PAGE analysis of the measles virion has confirmed the presence of six major viral proteins as well as a number of minor proteins. The major viral proteins include: large protein (L, 180-200,000 MW.); hemagglutinin protein (H, 80,000); polymerase protein (P, 72,000); nucleoprotein (NP, 60,000); hemolysin protein (F, 60,000; which is cleaved immediately following synthesis to yield F_1 + F_2); and matrix protein (M, 37,000). In addition, there has been brief mention of a seventh viral coded protein, the S protein (15,000). This protein seems to be a short-lived primary translation product, the significance of which is not yet known (Wechsler and Fields, 1978; Rima and Martin, 1979).

The presence of actin protein in association with measles virus has been noted by several researchers over the past few years. The incorporated actin protein, which is thought to be cellularly-derived, is known to migrate just above the F_1 protein of measles virus on SDS-PAGE analysis (Wechsler and Fields, 1978; Tyrrell and Norrby, 1978; Graves, Silver and Choppin, 1978; Rima and Martin, 1979; Stallcup, Wechsler and Fields, 1979).

The nucleoprotein of measles virus is the most abundant of the virion proteins. It is a phosphorylated protein,

which, together with the viral RNA, forms the nucleocapsid structure of the virus. This structure is associated with the P and L proteins to form the transcriptase complex of the virion. On isolation of the measles nucleocapsid structure, Waters and Bussell (1974) found it to be morphologically similar to the nucleocapsid structures of other paramyxoviruses such as Sendai virus, Newcastle disease virus, simian virus 5 and canine distemper virus. Waters and Bussell (1974) reported the RNA content of the nucleocapsid structure to be 5.12% for measles virus, 5.36% for canine distemper virus and 4.91% for simian virus 5.

Isolated nucleoproteins from various paramyxoviruses have been found susceptible to varying forms of restricted proteolytic cleavage. After purification of simian virus 5, Newcastle disease virus and Sendai virus via the use of trypsin, a single, smaller nucleoprotein was isolated (Mountcastle, Compans, Caliguiri and Choppin, 1970; Mountcastle, Compans, Lackland and Choppin, 1974). Waters and Bussell (1974) reported fragmentation of the measles nucleoprotein (61,000) and canine distemper nucleoprotein (54,000) after trypsin was used for virus purification. In these cases, however, two nucleoprotein segments of 38,000 and 24,000 were noted for measles virus and two segments of 27,000 and 22,500 were isolated from canine distemper virus.

The uncleaved nucleoprotein of simian virus 5 was found to differ in its degree of hydrophobicity relative to the cleaved form of the protein (Mountcastle et al., 1974). The

uncleaved nucleoprotein could not be suspended in an aqueous medium whereas the cleaved form was easily suspended and thus more hydrophilic. Amino acid analysis of the cleaved residues showed that they did not possess the overall degree of hydrophobicity to account for the behavior of the uncleaved nucleoprotein. To account for such hydrophobicity, Mountcastle et al. (1974) proposed that the more hydrophobic residues of the protein were concentrated on the external surface of the protein and would thus be susceptible to proteolysis. Absence of these residues could account for the unfolding of the nucleocapsid structure and the subsequent inflexibility since, after purification with trypsin, possible hydrophobic bonding sites on the nucleoprotein would be missing. These hydrophobic portions on the nucleoprotein are thought to play a crucial role in the assembly of the virion (Mountcastle et al., 1974). Measles virus and canine distemper virus nucleoprotein was cleaved into two proteolytic subunits after a similar trypsin purification procedure. This is unlike the results seen for the other paramyxoviruses. Nevertheless, the similar morphological characteristics of the various paramyxovirus nucleocapsid structures allow analogies to be drawn concerning the possible structure and function of the measles virus nucleoprotein.

Mountcastle et al. (1974) and Waters and Bussell (1974) postulated that spontaneous intracellular cleavage of the nucleoprotein may explain, in part, the developement of

persistance with respect to viral infections. *In vitro* cleavage is known to yield rigid inflexible nucleocapsid structures which are unsuitable for incorporation into mature virions. A similar *in vivo* situation could occur in a persistant disease such as SSPE (Mountcastle et al., 1974; Waters and Bussell, 1974). In this disease, larger than normal intracytoplasmic inclusion bodies containing nucleocapsid material have been noted in infected cells.

By analogy with other paramyxoviruses, the responsibility for transcription within the measles virion lie with the phosphorylated P protein and the L protein (Wechsler and Meissner, 1982). Pulse-chase experiments have shown that the P protein undergoes a time-distribution change during the progression of the infection. In the earlier stages of the infection, the distribution of the P protein between the nucleus and the cytoplasm is equal. Later, however, the P protein is found to be associated more with the nucleus than with the cytoplasm (Wechsler and Fields, 1978). Continued studies on both the P and L proteins are necessary before further knowledge concerning their roles in viral transcription can be understood (Rima, 1983). With respect to the L protein such experiments are tedious due to difficulties in isolating this protein from host cell contaminants and polypeptide aggregates possessing similar characteristics.

The M (matrix) protein, smallest of the measles proteins, is a non-glycosylated, hydrophobic protein

synthesized in the cytoplasm by membrane free ribosomes. The M protein in the measles virion, as well as in other paramyxoviruses, is protected from proteolytic digestion in whole virion preparations, thus implying that it is an internal protein (Shimizu and Ishida, 1975; Tyrrell and Norrby, 1978). Pulse-chase experiments have shown that Sendai virus M protein rapidly associates with cellular membrane fractions rather than remaining free in the cytoplasm. The rapidity of the association was such that it occurred before the chase period of the experiment started. However, a lag period was noted in the chase from the plasma membrane into the mature virion. It was suggested that some form of modification or restructuring of the membrane in the plane of the maturing viral envelope was taking place (Bowen and Lyles, 1982).

The affinity of the M protein for both the nucleoprotein and glycoproteins of paramyxoviruses is well documented. It was observed that Sendai virus nucleocapsid structures, isolated after a chloroform-methanol extraction procedure, possessed a brush border (Yoshida, Nagai, Yoshii, Maeno, and Matsumoto, 1976). Electron microscopic examination of the brush border showed that it was composed of a monolayer of small globular particles. The width of the nucleocapsid structures which possessed a brush border was reported as 33 nm as opposed to 20 nm for those which did not possess a brush border. Two dimensional immunodiffusion tests on the structures which were 33 nm in width confirmed

the presence of the M protein. These results suggested that the small globular particles bound to the nucleocapsid were made up of M protein (Yoshida et al., 1976).

In vitro experiments, using reassembled Sendai virus envelope particles, were done to examine the functional role of the M protein (Yoshida et al., 1976). These envelope particles, which contained viral glycoproteins and cell membrane lipids, possessed hemolytic, hemagglutinating and cell fusion activities. Results demonstrated the presence of the nucleoprotein in active hemagglutinating fractions, but only if those fractions also possessed the M protein. This suggested that the M protein acted as a mediator for the association of the nucleoprotein and glycoproteins of the virus (Yoshida et al., 1976). A similar function has been reported for the M protein of Newcastle disease virus. This protein was isolated from smooth membrane fractions capable of hemadsorption. These smooth membrane components are thought to be precursors of the mature viral envelope (Nagai, Ogura and Klenk, 1976).

The synthesis and assembly sequence of the M protein in the measles virion has previously been based on analogy from results seen with other paramyxoviruses. Data on the M protein of measles virus has thus far confirmed these analogies. It has been noted that a strong association between the nucleoprotein and M protein exists since, measles virus nucleoprotein preparations could not be prepared free of M protein contamination (Stallcup et. al.,

1979). The brush border previously described for Sendai virus has also been reported for intracytoplasmic measles nucleocapsids. Intranuclear measles nucleocapsids, which appeared 48-72 hours postinfection, did not appear to possess this brush border. As well, no M protein was found in the nucleus of measles infected cells (Dubois-Dalcq, 1979; Tyrrell, Rafter, Orvell and Norrby, 1980).

The role of the M protein in viral assembly has been hypothesized using data acquired on the properties and interactions of this protein with other paramyxovirus proteins. Portions of the nucleoprotein which are known to be hydrophobic and sensitive to proteolytic enzymes are thought to be functional sites for hydrophobic bonding with the M protein (McSharry, Compans, Lackland and Choppin, 1975). In this way the brush border appearance due to the small globular M protein bound to the nucleocapsid could be explained. The flexibility of the nucleocapsid and its tertiary folds may be linked to the presence of the M protein and its ability to form hydrophobic bonds.

Interaction of the M protein with the viral glycoproteins has been postulated as a major step in triggering viral assembly at the plasma membrane. The incorporation of the M protein into the maturing viral envelope is thought to be initiated by the appearance of its precursor, smooth hemadsorbing membranes containing viral glycoproteins (Nagai et al., 1976). In these membranes, viral glycoproteins are known to be randomly distributed and

highly mobile. However, in the mature viral envelope, the opposite is the case. Is the resultant aggregation and low degree of mobility of the glycoproteins in the viral envelope due to the incorporation of the M protein, or is it a precondition for the binding of the M protein to the glycoproteins of the envelope (Nagai et al., 1976)?

Researchers have continued to observe a highly ordered arrangement of nucleocapsid structures beneath specific aggregations of spike glycoproteins on the surface of paramyxovirus infected cells. Such observations as well as major characteristics of the disease subacute sclerosing panencephalitis support the hypothesized key role of the M protein in mediating viral assembly. Brain cells from infected patients are known to contain larger than normal quantities of nucleocapsid material. The material is poorly aligned along the plasma membrane and is naked in appearance. The cells appear to be in a non-productive state since no viral buds are seen on the surface of infected cells (Wechsler and Meissner, 1982).

Generally, sera from SSPE patients showed a significantly reduced response to the M protein relative to sera from patients with an acute measles virus infection. SSPE antisera levels against the other measles virus proteins were found to be normal. Dilutions of SSPE sera did not result in precipitation of the M protein and no inhibiting factors were found when tested with anti-M sera from other sources (Wechsler and Meissner, 1982). Thus, a

defective immune response, antibody excess and the presence of inhibiting factors were eliminated as reasons for the decreased antibody levels against the M protein. Further reports noted that: i) SSPE sera precipitated less M protein from wild type and SSPE strains of the virus; ii) convalescent sera from individuals precipitated M protein from both wild type and SSPE viruses equally well; iii) rabbit prepared antisera against five SSPE and two wild type strains precipitated M protein from each wild type and SSPE strain used. Thus it seemed that the reduced amount of anti-M antibody in SSPE patients was due to a defect in the synthesis of the antigen - the M protein - rather than by a defect in the host's immune response (Wechsler and Meissner, 1982).

This theory is supported by an *in vivo* case study and in the situation seen in two persistently infected SSPE cell lines, the IP-3 and Biken cell lines. In these two cell lines, mRNA for each measles protein, including the M protein, was found. In all three situations, each measles protein except the M protein was found. The absence of the protein may involve a defect in mRNA translation or be due to an immediate breakdown of the protein in the cytoplasm (Wechsler and Meissner, 1982). Nevertheless, the lack of M protein in the infected cell resulted in the abundance of smooth nucleocapsids randomly scattered throughout the cytoplasm. Without this protein, assembly of the virus cannot proceed and thus a persistant state is allowed to

develop. The significance of the mediating function of the M protein can thus be seen more clearly.

In the mature virion, the phospholipid bilayer structure of the plasma membrane is well preserved. Cellular proteins which are embedded in the plasma membrane are displaced by viral proteins, which in the case of measles virus are the hemagglutinin (H) and fusion (F) glycoproteins. These glycoproteins are responsible for adsorption to and fusion of host cells, respectively.

The synthesis of both viral glycoproteins as well as other cellular membrane and secretory proteins takes place on membrane bound ribosomes in the cytoplasm. A signal sequence on the nascent polypeptide is inserted into the signal recognition particle located on the rough endoplasmic reticulum. This insertion allows for subsequent translocation of the growing glycoprotein across the lipid bilayer of the membrane. Signal sequences are thought to be composed of 16-20 amino acid residues. These sequences are cleaved off as the protein is extruded into the lumen of the rough endoplasmic reticulum (Meyer, 1982; Walter and Blobel, 1982). The continued addition of amino acids to the polypeptide and the folding of the protein into its tertiary structure act as driving forces to move the polypeptide across the membrane. When the termination signal on the mRNA is reached, the membrane bound ribosomal subunits dissociate leaving the carboxyl terminal portion of the protein on the cytoplasmic side of the membrane. At this point, the protein

remains as it is, embedded in the membrane as a transmembrane protein. Such proteins possess a hydrophobic domain of 20-25 amino acids which span the membrane. On either side of the membrane are hydrophilic domains. A small 30-40 amino acid hydrophilic domain containing the carboxyl terminal is located on the cytoplasmic side of the membrane. The largest domain, consisting of the remainder of the protein, protrudes into the lumen of the rough endoplasmic reticulum (Lodish, Zilberstein and Porter, 1981). Post-translational modifications occur as the protein folds on itself and, as it is transported through the cell to the plasma membrane.

Evidence for the transmembrane nature of the glycoproteins of Sendai virus was reported by Bowen and Syles (1981). By exposing the cytoplasmic surface of the plasma membranes of infected cells to trypsin, while protecting the external surface, it could be shown that a molecular weight loss of 1000-2000 occurred for each protein. This would only occur if a portion of these proteins was exposed on the cytoplasmic surface of the membrane.

The hemagglutinin and fusion glycoproteins resemble spikes which protrude from the outer surface of the viral envelope. By subjecting whole virion preparations to a 0.1% trypsin solution (a proteolytic enzyme), the hemagglutinin spikes could be removed. However, a 0.25% trypsin solution was required to remove the fusion protein (Stallcup et al.,

1979). This implied that the fusion protein was more closely associated with the bilayer structure of the envelope than were the hemagglutinin spikes. It was later found that anti-hemagglutinin serum failed to react with whole virion preparations after exposure of the virion preparation to a 0.004% trypsin solution. However, the reaction of the antifusion serum was not affected. On the other hand, acetone treatment destroyed the functional and antigenic capabilities of the fusion protein, but not of the hemagglutinin protein (Armstrong, Fraser, Dermott and Shirodaria, 1982). It was thus concluded that the fusion protein was intimately associated with the lipid component of the envelope. Since it is known to possess antigenic capabilities, it must therefore have a hydrophilic portion exposed on the exterior surface of the virion (Armstrong et al., 1982).

The fusion protein of paramyxoviruses undergoes a post-translational cleavage to form the F_1 and F_2 subunits. These two subunits remain linked by disulphide bonds to form the active fusion protein. The F_1 portion contains the carboxyl terminal of the F protein and the F_2 portion contains the amino terminal (Scheid and Choppin, 1977). This functional F complex accounts for the fusion of host cell membranes (syncytia formation), which is a pathological finding of measles virus infection, and for lysis of these cells. Radiolabelling experiments have proven that the entire carbohydrate portion of the protein is associated

with the F_2 subunit (Hardwick and Bussell, 1978; Tyrrell and Norrby, 1978; Graves et al., 1978). This is unlike the situation seen for other paramyxovirses in which both the F_1 and the F_2 subunits possess carbohydrate moieties. However, significantly more carbohydrate is associated with the F_2 subunit than with the F_1 subunit in each case (Scheid and Choppin, 1977; Hall, Lamb and Choppin, 1980). It is postulated that the carbohydrate groups bound to the F_2 protein may play a role in limiting the accessibility of the cleavage site on the parent F protein. This would aid in the cleavage of the protein at a specific site so that the biological activity of the protein complex would be further ensured. It is also known that the remainder of the polypeptide chain is relatively resistant to any further proteolysis (Scheid and Choppin, 1977). Thus, it is unlikely that any further proteolytic digestion of the fusion protein would occur.

The importance of the fusion protein in the infection process of measles virus was noted in the late 1960's by the failure of the standard formalin killed vaccine to prevent measles infection. A significant percentage of children who had received this vaccine later developed atypical measles virus infection on exposure to the virus. It was later found that formalin treatment destroyed the fusion protein's immunogenic capabilities. The hemagglutinin, however, remained immunogenic. Transmission of the virus during subsequent infection took place by fusion of infected cells

with adjacent uninfected cells. Thus an atypical measles virus infection was allowed to take place in the face of a secondary immune response to all the other measles proteins except the fusion protein (Choppin, Richardson, Merz, Hall and Scheid, 1981).

The hemagglutinin protein is essential for the survival of the virus since it is responsible for attachment of the virus to host cells suitable for viral replication. In the case of Sendai virus, Newcastle disease virus and simian virus 5, the hemagglutinin protein shares its spike projection with the neuraminidase entity of these viruses. In the Morbillivirus genus however, no neuraminidase is found. The hemagglutinin protein of measles virus is a transmembrane protein projecting from the viral envelope on a single spike. Its synthetic pathway in infected cells has recently been shown to follow that of cellular glycoproteins as described previously (Bellini et al., 1983)

With the exception of the hemagglutinin protein, the proteins of measles and canine distemper virus are antigenically similar. Antiserum against measles hemagglutinin was able to precipitate both measles and CDV hemagglutinin. Canine distemper hemagglutinin antiserum was able to precipitate its own hemagglutinin but not that of measles. Two-way immunoprecipitation crosses were noted for the other proteins of both viruses. It was hypothesized that the hemagglutinin protein would be under the greatest selective pressures relative to the other proteins since it

must be adapted for adsorption onto different cell types suitable for viral replication. These selective pressures may permit changes in the antigenic determinant of the H protein and thus be responsible for the differences in immunoprecipitation of this protein relative to the others (Hall et al., 1980). It has also been noted by numerous research groups that canine distemper virus hemagglutinin is not a biologically active hemagglutinin whereas that of measles virus is (Campbell, Cosby, Scott, Rima, Martin and Appel, 1980; Hall et al., 1980). Again, various selective pressures may have caused this difference.

The rate of migration of the hemagglutinin polypeptides through polyacrylamide gel matrixes differs according to the amount of post-translational processing they have undergone (Stallcup et al., 1979). Hemagglutinin, purified from whole virions, migrated as a broad fuzzy band, whereas if purified from cell membrane extracts , it migrated as a more compact and slightly faster moving band. The difference in the migration rates was thought to be linked to the greater number of carbohydrate groups associated with the complete glycoprotein relative to the incomplete form found in the various cell membranes (Stallcup et al., 1979). Further structural studies have demonstrated that the hemagglutinin protein contains intermolecular disulphide bonds which may play a role in forming the active protein unit. The hemagglutinin-neuraminidase complex of Sendai virus and Newcastle disease virus have been found in dimeric or

tetrameric and dimeric forms respectively (Markwell and Fox, 1980). Lund and Salmi (1981) have purified the measles hemagglutinin protein as a dimer structure using non-reduced and reduced SDS-PAGE analysis. Presently, it is thought that this dimer structure is the natural form found in infected cells and virions (Casali, Sissons, Fujinami and Oldstone, 1981; Rima, 1983).

Activity of the hemagglutinin protein has been found to differ amongst various strains of measles virus. Several strains have been shown to have no hemagglutinating activity when tested with monkey red blood cells in the presence of phosphate buffered saline. However, in the presence of 0.8M ammonium sulfate, hemagglutinating activity could be recovered. It was thus refered to as salt dependent hemagglutination (Shirodaria, Dermott and Gould, 1976). Some SSPE strains, specifically the Lec and SSPE(I) strains, possess both non-salt and salt dependent hemagglutinating particles. With reference to the Lec strain, two plaque variants were grown, small and large. The small plaque variant possess the salt dependent hemagglutinin particle only, whereas the large variant possess both types of hemagglutinin particles. The centers of the large plaques contained non-salt dependent particles while the peripheral edges of the plaques contained predominantly salt dependent particles. It was thus postulated that the salt dependent particles may be precursors for the fully active hemagglutinin protein (Gould, Cosby and Shirodaria, 1976).

Continued research on the hemagglutinin protein of various measles and SSPE strains revealed a number of unique characteristics. Hemagglutinin protein isolated from a lytic Lec SSPE strain was found to be in the form of a dimer whereas the same protein isolated from a persistant Lec SSPE strain was in the form of a monomer (Stephenson, Siddell and ter Meulen, 1981).

An SSPE isolate recovered by co-cultivation with Vero cells was found to be defective in the synthesis of its hemagglutinin protein (Wild, Giraudon, Bernard, and Huppert, 1979). Hemagglutination tests performed on this isolate using Vervet red blood cells proved negative. As well, freeze thawed cell extracts of infected cells injected into guinea pigs did not elict an antibody response to the hemagglutinin protein even though anti-fusion and neutralizing antibodies were detected. SDS-PAGE analysis of radiolabelled virions did not reveal the presence of a hemagglutinin protein in these virions. It was proposed that this SSPE isolate either did not synthesize the hemagglutinin protein or that it was broken down immediately after synthesis by cellular enzymes (Wild et al., 1979).

As well as structural differences, major immunological variations were noted in the nature of the hemagglutinin protein found in persistant and lytic or wild type strains. The use of monoclonal antibodies directed against the hemagglutinin protein of the wild type Edmonston strain demonstrated that three different Lec SSPE strains as well

as the Hu-1 and Hu-2 strains of measles lacked the same hemagglutinin antigenic determinants associated with the Edmonston strain (Trudgett, Gould, Armstrong, Mingioli and McFarlin, 1981). Comparable antigenic differences were noted between the hemagglutinin of a Lec SSPE strain from a persistently infected cell line and the hemagglutinin from a lytic Lec and a wild type Edmonston strain (ter Meulen, Löffler, Carter and Stephenson, 1981). Further studies by this group using radiolabelled binding assays, viral neutralization and hemagglutination inhibition assays were carried out. They were able to demonstrate that significant differences existed in the antigenicity of the hemagglutinin polypeptide structure, as well as in the distribution, location and affinity of the biologically active portions of this glycoprotein. These differences concerned the hemagglutinin protein of wild type measles strains (Edmonston, Woodfolk and EVA), known SSPE strains (Mantooth, Halle and Lec) and an isolate from acute measles encephalitis - the Braxato strain. Generally, no common pattern differentiating the hemagglutinin isolated from an SSPE strain or a wild type strain of virus could be found. Isolates were noted in which a given monoclonal antibody series cross-reacted well with a given measles or SSPE hemagglutinin. Even though antigenic determinants could be found that were shared amongst some viral strains (McFarlin, Bellini, Mingioli Behar, and Trudgett, 1980), distinct structural changes resulting in topographical and

immunological differences were present amongst other strains (ter Meulen et al., 1981). A series of 5 monoclonal antibodies, each with its own specificity against the activity of the Edmonston hemagglutinin protein, were used to map 3 epitope regions on the surface of this polypeptide. These regions all traversed the active site of the hemagglutinin in such a way that one region overlapped the other two which were distinct from one another (Carter, Willcocks, Löffler and ter Meulen, 1982). Thus, structural variations within the hemagglutinin active site are being noted as research into this area expands.

It would seem that the selective pressures exerted on the viral genome are sufficient to cause alterations in the gene products such that each strain of virus could possess a unique hemagglutinin protein. The end result of these alterations could be the ability of the virus to adsorb to numerous different cell types in the body. In this way, a virion possessing newly acquired capabilities to bind to central nervous system tissue could replicate in the CNS, assuming it is able to gain entry. Replication of that virus and the subsequent destruction of cells in this vital system is the situation seen in subacute sclerosing panencephalitis and possibly in multiple sclerosis.

A 43,000 mw protein associated with numerous enveloped viruses, and possibly required in viral assembly, is found repeatedly on SDS-PAGE analysis of purified virions. This protein comigrates with purified actin preparations on

SDS-PAGE analysis and it has been associated with immune precipitates of viral proteins (Wang, Wolf, Lamb, Choppin and Goldberg, 1976; Tyrrell and Norrby 1978). This 43,000 protein was found to be the only measles associated protein to contain a radiolabel after growth of the virus in pre-radiolabelled Vero cells. It was concluded that the protein was cellularly derived actin that had been incorporated into the maturing virion (Tyrrell and Norrby 1978). However, the role of actin in viral assembly is not clearly understood (Giuffre, Tovell, Kay and Tyrrell, 1982). It may be involved in some form of transmembrane communication between the envelope, M and nucleocapsid structures since co-capping of these viral components at measles virus budding sites has been observed. In the presence of cytochalasin B, an inhibitor of actin polymerization, only co-capping of the envelope and M proteins were seen. The nucleocapsid inclusions tended to remain in the cytoplasm of the cell in this case. It seemed that the linkage, possibly by actin filaments, which guided and aligned the nucleocapsids with the viral budding sites, was not functional in the presence of cytochalasin B (Tyrrell and Ehrnst, 1979). Somewhat opposed to these results are those of Griffin and Compans (1979). This group found no requirement for an intact cytoskeletal network for the maturation of influenza virus as viral budding was noted in the presence of cytochalasin B. Further studies on the effect of cytochalsin B on measles virus maturation

demonstrated that the inhibition of actin polymerization could be rapidly reversed simply by removal of the drug (Stallcup, Raine and Fields, 1983). To minimize the drug induced inhibition of protein glycosylation, cytochalasin B was added to infected cultures 2 hours prior to harvesting the virus. Yields of released virus were 88% to 99% less than that seen for the untreated cells. The yield of cell associated virus was 8 fold greater than seen in the untreated cells. After exposure to the drug for 46 hours, the yields of cell-associated virus were equal to or greater than that for the control yields, whereas the yields of released virus were 100 to 1000 fold less. It seemed that the drug worked primarily to inhibit viral release rather than to inhibit synthesis of viral components. SDS-PAGE analysis confirmed the presence of each of the measles virus proteins purified from infected cells exposed to cytochalasin B. It was thus hypothesized that actin microfilaments may be acting in a manner similar to their actions during cell division - by contraction of the microfilaments to cause cleavage of the viral bud from the cell membrane (Stallcup et al., 1983). Nevertheless, actin is still found in purified measles virus preparations as well as in other viral preparations. If a role for actin in viral assembly is proven, then its presence would be more clearly understood.

Recently, a seventh viral encoded protein, the "S" protein has been reported in both measles (Wechsler and

Fields, 1978; Rima and Martin, 1979) and canine distemper virus (Campbell et al., 1980). The protein is nonglycoslated and thought to be a short lived primary translation product since it turns over rapidly during viral synthesis.

The advent of high performance liquid chromatography (HPLC) as an analytical tool has allowed researchers in numerous fields to accurately determine various characteristics of unknown samples. These characteristics may be studied through the use of different chromatographic columns, ie; molecular exclusion, ion exchange or bonded reverse phase columns. Molecular exclusion columns act as filter systems for the determination of molecular size. Cation or anion exchange columns, (depending on the ionic group bonded to the resin beads), separate components in a solution according to their avidity for the ionic groups bonded to the resin beads. The bonded reverse phase columns will separate sample components such that the most polar solutes elute first.

High performance liquid chromatography using bonded reverse phase columns offer advantages to this study which other analytical systems do not possess. The high resolution afforded high performance liquid chromatography enables detection of minor differences in the properties of the solute, which, in this case, are tryptic peptides. With minor exceptions to the M protein, differences in one dimensional tryptic peptide profiles could not be demonstrated after limited proteolysis of proteins from

measles and SSPE linked strains. Conclusions accounting for variations in biological activity and strain origin could not be made for any of the isolates studied by this method (Rima, Roberts and Martin, 1983). Bonded reverse phase high performance liquid chromatography uses octadecylsilane (C_{18}) chains which are covalently bonded to silica based beads. This type of bead construction is very durable and can be reused many times before any appreciable loss in resolution occurs. Accuracy, reproducibility and sensitivity are another series of advantages offered by reverse phase HPLC systems, which, in this case are important considering that only small sample sizes are available. A system capable of detecting small quantities of minute components must be used. Analysis times are dramatically shortened using this method (Kratzin, Yang, Götz, Thinnes, Kruse, Egert, Pauly, Köbel, McLaughlin and Hilschmann, 1983). The desired result can be interpreted less than an hour after the sample is put onto the system. This compares to a day or more for other analytical systems. Lastly, the compatibility of this method for automation and computerization is very important in this study. To carry out the desired comparative studies on the hemagglutinin protein of different measles and SSPE strains, it would be very practical to make use of a computer system to avoid intrepretive errors seen in other methods.

The bead structure used in reversed phased high performance liquid chromatography forms the non-polar stationary phase of the chromatographic system. Elution of

peptides from the column is carried out using a polar solvent gradient of increasing concentration. The peptides will elute in order of increasing hydrophobicity. Acetonitrile, an organic polar solvent, was used as the mobile phase of the chromatographic system. Methanol, the alternative solvent, was not used due to its high viscosity on mixing with water and its lesser efficiency as an eluant. The structure of the stationary phase of reverse phase columns necessitates the use a hydrophobic ion pairing agent. Such agents have a two-fold purpose in these gradients. Firstly, they combine with free silanol groups in the stationary phase to prevent irreversible binding of peptides to the column. Secondly, their addition to the solvents lowers the pH which promotes protonation of acidic peptides. This results in a more efficient interaction with the functional groups of the stationary phase (Bennett, Browne and Solomon, 1980). In this case, trifluoroacetic acid was used.

In this particular study, the Hallé strain of measles virus is of interest. This strain, a known SSPE isolate, was isolated from lymph node tissue of a SSPE patient. It is also capable of readily establishing persistant infection in numerous cell lines (McKimm-Breschkin, Breschkin and Rapp, 1982).

Growth of this strain at 37°C results in the production of defective interfering particles (Tsang, Chang and Marusyk, 1981; McKimm-Breschkin et al., 1982). The viral

plaques produced at 37°C consisted of alternating rings of dead and living cells. They were thus referred to as ring plaques. It was postulated that the production of defective interfering particles was linked to the development of these ring plaques (Tsang et al., 1981).

The Hallé strain is characterized by low hemagglutination titres similar to some other known SSPE isolates, ie: Berg and AIK, but unlike that seen for the Lec SSPE and wild type Edmonston strains. On electron microscopic examination of the Hallé particle, hemagglutinin peplomers could not be visualized on the surface of the viral envelope. SDS-PAGE analysis of these virions however, revealed the presence of a possible hemagglutinin protein and the virus was found to be infective if stocks were grown at 32°C (Marusyk, R.G. and Tyrrell, D.L.J., 1983). Considering the numerous variations in the nature of the hemagglutinin protein of various measles and SSPE virus strains, it would be of value to: i)further investigate the significance of the properties characteristic of the hemagglutinin protein of the Hallé SSPE strain and; ii) consider the possible role this protein may have in the development of a persistant SSPE infection.

The results at present indicate that the hemagglutinin protein of Hallé strain is synthesized during viral replication. Infectivity at 32°C and the production of ring plaques at 37°C by this virus imply that the virus is able to adsorb to host cells. However, as with some other known

SSPE strains, hemagglutinin titres are much lower relative to the Lec SSPE and Edmonston wild type strains. Such differences in the biological activity of the hemagglutinin protein in various measles and SSPE linked strains bring to question the structure-function relationship of the active site(s) of this protein. Structural changes within the active site(s) of the Hallé hemagglutinin protein relative to other high hemagglutinin titre strains may account for the biological differences characteristic of this strain. To determine the nature of any structural differences, comparative analysis of hemagglutinin tryptic peptides from the Hallé strain and other measles-SSPE related strains can be done. To satisfy the need for accurate and rapid analysis of these peptides, reversed phase high performance liquid chromatography has been used to separate the peptides. Computer analysis of the resulting chromatograms will enable a precise reproducible comparison of the strains to be carried out. This will hopefully provide insight into how a particular strain of measles virus is able to adsorb to central nervous system tissue and thus cause SSPE and possibly multiple sclerosis.

II. Methods and Materials

A. Tissue Culture

Vero (African Green Monkey Kidney) cells obtained from Flow Laboratories, Inglewood , CA., were used in this study. The cells were grown in glass culture bottles (Blake bottles) until 100% confluency was reached. Passage of the cells involved rinsing the cell monlayer in 20 mL of phosphate buffered saline (PBS) and then incubating at 37°C with 2 mL of trypsin (Difco, Canlab Distributors, Edmonton, Alberta). Incubation continued until the cells detached from the glass surface. The cells were then suspended in 10 mL of minimal essential media (MEM) and redispersed in 6 mL aliquots into 120 mL of MEM. Roller bottles used for the growth of cells were coated with 3 mL of fetal calf serum for at least 3 h prior to the addition of the cells. This facilitated adherence of the cells to the glass surface.

B. Growth Medium

The growth medium used was Eagle's modified minimal essential medium (Flow Laboratories, Inglewood, CA.). The medium was sterilized by autoclaving at 120°C, and 15 psi. for 20 min. Into 500 mL aliquots of MEM were added the following i) 5 mL of an antibiotic solution containing penicillin G sodium (100 IU/mL, final concentration) and streptomycin sulfate (100 IU/mL, final concentration); ii) 5 mL of a 0.2 M glutamine solution; iii) 6.5 mL of a 7.5%

sodium bicarbonate solution; iv) 10 mL of donor calf serum. The additives were all filter sterilized prior to use. The donor calf serum (Flow Laboratories, Inglewood, CA.) was inactivated by incubation at 60°C for 30 min.

C. Virus

The Hallé and Edmonston strain of measles virus were both obtained from the American Type Culture Collection. The Hallé strain was isolated from lymph node biopsies from a 12 year old female with clinical SSPE symptoms. The Edmonston strain was isolated from a patient during the acute phase of a typical measles virus infection.

D. Viral Growth

When the cell monolayers were 80-90% confluent, infectious virus was added to the cells. A 0.1 mL aliquot of infectious virus material was diluted (in 100 mL of MEM) to give a multiplicity of infection of 0.1. Each culture was then incubated with 10 mL of this solution for 2 h at 37°C to allow for virus adsorption to the cells. 50 mL of MEM was then added to the cultures which were then incubated at 32°C.

E. Viral Harvest and Purification

When complete cytopathic effect was observed in the infected cultures, the cells were removed from the glass surface by vigorous shaking and then placed at -35°C for

freeze-thawing. After thawing, the suspension was centrifuged in a Sorval GSA rotor (Dupont Instruments, Wilmington, DL.) for 20 min at 11,390 x g. The supernatant was removed and pooled so that extraction of the viral protein could be carried out. Extraction was accomplished by dissolving 20 g sodium chloride and 80 g polyethylene glycol (PEG 8000) per litre of supernatant and incubating the mixture at 4°C overnight. The solution was then centrifuged in a Sorval GSA rotor for 20 min at 11,390 x g to pellet the viral material. After removal of the supernatant, the virus pellet was resuspended in 5-10 mL of Tris-sodium chloride-EDTA buffer (TNE; 0.2490 M Trizma base, 1.0 M sodium chloride and 0.0127 M EDTA, pH 7.2). The pooled suspensions were then layered onto a discontinuous sodium-potassium tartrate gradient (50%, 40%, 30%, 15% sodium-potassium tartrate in TNE). The gradient was centrifuged at 104,000 x g in a Beckman SW28 rotor (Beckman Instruments, Palo Alto, CA.) for 2.5 h. Following centrifugation, the viral band on the gradient was extracted, dialyzed against TNE buffer and recentrifuged under the same conditions. The viral band was then removed from the second gradient, dialyzed against Tris-glycine buffer (TG; 0.0500 M Trizma base, 0.3834 M glycine, pH 8.2) and then stored at -20°C for later analysis.

F. Purification of the Hemagglutinin Protein

Purified viral material was sonicated at 6 KHz on a Branson Sonifier Cell Disrupter 185 (Branson Sonic Power, Danbury, C.) until the sample appeared translucent. The non-ionic detergent, Triton X-100 (Fisher Scientific Company, Edmonton, Alberta) was then added to a final concentration of 10%. The sample was incubated for 30 min at room tempurature and vortexed 3-4 times during this period. The mixture was then centrifuged at 370,000 x g in a Beckman SW60 rotor to pellet any insoluble material (the nucleocapsid, polymerase, L, and matrix proteins as well as any other cellular debris). The supernatant was removed and layered onto a sucrose-tartrate gradient (30%, 50% sodium-potassium tartrate in TNE over a 15% sucrose cushion in TG). This gradient was then centrifuged at 202,000 x g in a Beckman SW40 rotor for 2 h. The top layer of the gradient was removed and stored at 4°C until further purification. The remaining portion of the gradient was fractionated into 1 mL aliquots to analyze for the presence of any insoluble material.

The stored supernatant material was layered onto a 1 x 40 cm QAE-Sephadex A25 anion exchange column (Pharmacia Fine Chemicals Inc., Dorval, Que.). The sample was eluted from the anion exchange column using a linear 0-0.5 M sodium chloride gradient in TG buffer. Column fractions were monitored and collected in 1 mL aliquots using a LKB 2089 Uvicord III detector (280 nm), a LKB 2210 1-Channel Recorder

and a LKB 2111 Multirac fraction-collector (LKB, Fisher Scientific Company, Edmonton, Alberta).

Fractions found to contain biologically active hemagglutinin by hemagglutination assay and SDS-PAGE analysis were pooled, concentrated and dialyzed against TG buffer. The sample was retreated with Triton X-100 (final concentration 10%) and layered on a discontinuous sucrose gradient (50%, 30%, 15% sucrose in TG). The gradient was centrifuged at 202,000 x g for 15 h in a Beckman SW40 rotor. Afterwards, the gradient was fractionated into 0.7 mL aliquots. Hemagglutination titers and UV absorbance results were recorded for each fraction. SDS-PAGE analysis was performed on those fractions showing active hemagglutination and/or high UV absorbance.

G. Detection of the Purified Hemagglutinin Protein

The hemagglutinin activity of each column and gradient fraction was determined using a microtitration technique (Cooke Engineering Company, Alexandria, Va., U.S.A.). Serial dilutions of 25 ul of each fraction collected were made in phosphate buffered saline (PBS). To each dilution was added 25 ul of a 1% suspension of *Ceropithecus aethiops* erythrocytes (Connaught Laboratories Ltd., Willowdale, Ont.). Hemagglutination assays were incubated at 37°C for 30 min. Ultraviolet absorbance readings at 280 nm (Gilford Instrument Lab. Inc., Oberlin, Ohio) were recorded for each fraction collected. The salt concentration of each fraction

was determined by measuring the electrical conductivity on a YSI Model 32 Conductance Meter (Scientific Division, Yellowsprings Instrument Co., Inc., Yellow Springs, Ohio).

The fractions containing the hemagglutinin protein were pooled, concentrated against polyethylene glycol 8000 and then dialyzed against TG buffer. A small sample from each of the pooled samples was placed on a SDS-PAGE gel to determine the purity of the hemagglutinin protein.

H. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide slab-gels were prepared according to a modification of the method described by Maizel (1971). The gels were prepared and run in a Bio-Rad Model 220 Vertical Slab Electrophoresis Unit. A 3% stacking gel, measuring 150 x 30 x 1.5 mm was formed on top of a 13% resolving gel (acrylamide-bisacrylamide ratio of 30:0.8). The resolving gel measured 150 x 90 x 1.5 mm. A slotted comb, placed into the stacking gel during polymerization, formed the required sample wells.

Protein samples for SDS-PAGE analysis were prepared as follows. Aliquots containing 0.12-2.0 ug of protein per 70 ul as determined by protein assay, were used. Dissociation buffer was added to the sample to a final concentration of 0.05 M Tris-HCl, pH 7.6, 1% SDS, 0.1% beta-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue. The samples were heated in a boiling water bath for 5 min prior to being placed on the gels. Electrophoresis was carried out for 160

min at 30mA per slab gel.

I. SDS-Polyacrylamide Gel Staining Procedures

a. Silver Staining Method

On completion of electrophoresis, the gels were stained with silver according to a modification of the method by Wray, Boulikes, Wray and Hancock, (1981). Upon removal from the electrophoresis unit the slab gels were soaked in a 50% methanol:10% acetic acid solution for 30 min. Following this, the slab gels were washed in 50% methanol for at least 6 h. After 3 h, the methanol wash was renewed and the gels were washed for the remaining time.

The silver staining solution was made up as follows: 1.6 g of silver nitrate (Fisher Scientific Company, Edmonton, Alberta) was dissolved in 8 mL of water. Into 38.2 mL of water was added 3.78 mL of 1.0 N sodium hydroxide (Canlab Distributors, Edmonton, Alberta). In addition to this, 0.4 mL of water and 2.4 mL of 28.0-30.0% ammonium hydroxide (North American Scientific Chemical, Edmonton, Alberta) were also added. The silver nitrate solution was then slowly added to the hydroxide solution until saturation was reached. Water was added to this solution to bring the total volume to 200 mL, and the gels were soaked in this staining solution for 20 min.

Following removal of the staining solution, the gels were washed twice water for 15 min each. The developing

solution was made immediately prior to use. This solution contained 0.25 mL of 37% formaldehyde (Fisher Scientific, Edmonton, Alberta) and 2.5 mL of 1.0% citric acid (Canlab Distributors, Edmonton, Alberta) dissolved in 500 mL of water. The gels were placed in the developing solution until the protein bands became distinguishable. To terminate development, the gels were placed in a 50% methanol:10% acetic acid solution.

During each step of the staining procedure the gels were keep in constant slow agitation so that they would not come in contact with the surface of the staining tray. A glass staining tray and highly purified water were used throughout the procedure.

b. Coomassie Blue Staining Method

SDS-PAGE gels were stained in 0.2% Coomassie Brilliant Blue R-250 prepared in a methanol:glacial acetic acid:water solution (5:1:4). The gels were stained overnight in this solution. The following day, the gels were destained until clear in the same solution without the Coomassie Brilliant Blue.

J. Protein Quantitation

Purified viral material and aliquots of purified hemagglutinin protein were analyzed for protein content. Protein assays were performed using the Bio-Rad assay system (Bradford, 1976). Bovine serum albumin was used as the

reference standard for each assay.

K. Iodination Procedure

The iodination procedure was carried as follows; 0.5 ml of hemagglutinin protein in PBS was placed in a tube containing 40 ug of Iodo-gen, (1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril; North American Scientific Chemical, Edmonton, Alberta). To this, 1.0 ul of ^{125}I (0.5 mCi; Radiopharmaceutical Center, Edmonton, Alberta.) was added. The mixture was then incubated at room tempurature for 2 min. This was followed by the addition of 0.5 ml of 0.1M potassium iodide (KI) to the reaction mixture. The entire reaction mixture was then removed from the reaction vessel, placed in another vessel and incubated on ice for 30 min. As the hemagglutinin preparation was being radiolabelled, 0.5 ml of a 0.1% BSA preparation was simultaneously iodinated using 0.5 ml of 0.1M unlabelled KI. After incubation of both reactions on ice, 100 ul of the iodinated 0.1% BSA mixture was added to the radioactive hemagglutinin mixture to act as a carrier protein.

The unreacted iodine was separated from the the bound iodine through the use of a Sephadex G25 gel exclusion column (0.5 X 18 cm). To minimize non-specific binding, 1.0 ml of 0.1% BSA was passed through the column followed by the radioactive sample. Fractions were collected in 0.5 ml aliquots.

L. Tryptic Digest of Hemagglutinin Protein

Fractions containing purified hemagglutinin protein were pooled, concentrated and dialyzed against 0.05 M ammonium bicarbonate. Aliquots containing purified hemagglutinin were enzymatically digested using L-tosylamide 2-phenyl ethyl chloroethyl ketone (TPCK)-treated trypsin (Worthington Chemicals, Freehold, N.J.). The enzyme:substrate ratio was 1:12.5(w:w). To minimize the effect of temperature and time, the digests were done simultaneously. The enzyme-substrate mixture was incubated at 37°C for 2 h in 0.05 M ammonium bicarbonate. To ensure maximal digestion of the hemagglutinin, one half of the trypsin was added at time 0 and the remaining portion was added one hour later. Following incubation, the digests were frozen at -20°C and then lyophilized overnight. The samples were then stored -20°C for later analysis.

M. HPLC Conditions

High performance liquid chromatographic analysis of the tryptic digests were performed using the following instruments: Varian 5040 liquid chromatograph, Varian UV-50 variable wavelength detector, Varian CDS 401 data processor (Varian Associates Inc., Walnut Creek, CA. U.S.A.) and a Waters uBondapak C18 reverse phase column (3.9mm x 30cm);(Waters Associates Inc., Milford, MA. U.S.A.). Digest samples were dissolved in 50 ul of the initial mobile phase and injected onto the column through a Rheodyne fixed volume

loop injection valve. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA; solvent A, pH 2.06; Fisher Scientific Company, Edmonton, Alberta), and 0.05% TFA in acetonitrile (solvent B; Fisher Scientific Company, Edmonton, Alberta). A linear gradient of 10% solvent B to 50% solvent B over a 50 minute time period was used to elute the sample from the column. A flow rate of 1 mL per min gave an average pressure of 100 atmospheres throughout the run. Detection by ultraviolet absorbance was done at 220 nm, which is in the absorbance range of peptide bonds. Full scale deflection was set at 0.0625 absorbance units.

N. Buffer Solutions

The buffer solutions used for this study were made according to tables listed by Gomori (1955).

O. Manuscript Production

This thesis was prepared using the Textform program of the Michigan Terminal System at the University of Alberta. The thesis was printed on a Xerox 9700 page printer.

III. Results

A. Purification of Measles Virus

Measles virus was purified by centrifugation on two consecutive discontinuous sodium-potassium tartrate density gradients. The purified viral material banded at the interface of the 40% and 50% layers of the gradients. The buoyant density at this point in the gradient has been reported to be 1.225 g/ml, (Tsang et al, 1981), and was confirmed in this study. The infectivity titres were 4 X 10^4 and 1 X 10^4 pfu/ml for the Edmonston and Hallé strains, respectively.

B. Purification of Measles Virus Hemagglutinin Protein

Purification of the hemagglutinin protein was accomplished by centrifugation and anion exchange chromatography. The procedure developed here is summarized in Figure 1. Centrifugation of the sonicated and detergent-treated virus sample at 485,000 x g pelleted insoluble cellular and viral components (Figure 1, step 1). As seen in Figure 2b, lane 3, the pellet contained a range of material of varying molecular weight. The pellet was retained for further analysis. Certain components which remained solubilized in the detergent-containing portion of the gradient could be partially removed by centrifugation on a sucrose-tartrate gradient (Figure 2b). The residual hemagglutinin activity noted in Figure 2b was observed in

Figure 1. Flow chart summarizing hemagglutinin purification procedure.

Purified viral material (2X Na-K-tartrate gradient)

STEP 1. a.Sonication and addition of Triton X-100 to 10%. Incubate at room temperature, 30 min.
b.Centrifuge at 485,000 x g, 90min.

STEP 2. Centrifuge supernatant on a sucrose-tartrate gradient, 285,000 x g, 2 h. Pellet retreated

STEP 3. a.Pass detergent portion of gradient through QAE-Sephadex A25 anion exchange column.
b.Hemagglutinin titre, UV absorbance and SDS-PAGE analysis of column fractions.

STEP 4. a.Pool hemagglutinin containing fractions, add Triton X-100 to 10%, incubated at room temperature, 30 min.
b.Centrifuge preparation on sucrose gradient, 5 h, 285,000 x g.

STEP 5. a.Pass detergent portion of gradient through QAE-Sephadex A25 anion exchange column.
b.Analyze fractions with active hemagglutinin and/or high UV absorbance by SDS-PAGE.

STEP 6. a.Treat hemagglutinating fractions with Triton X-100, pass through anion exchange column.
b.Hemagglutinin titre, UV absorbance and SDS-PAGE analysis of column fractions.

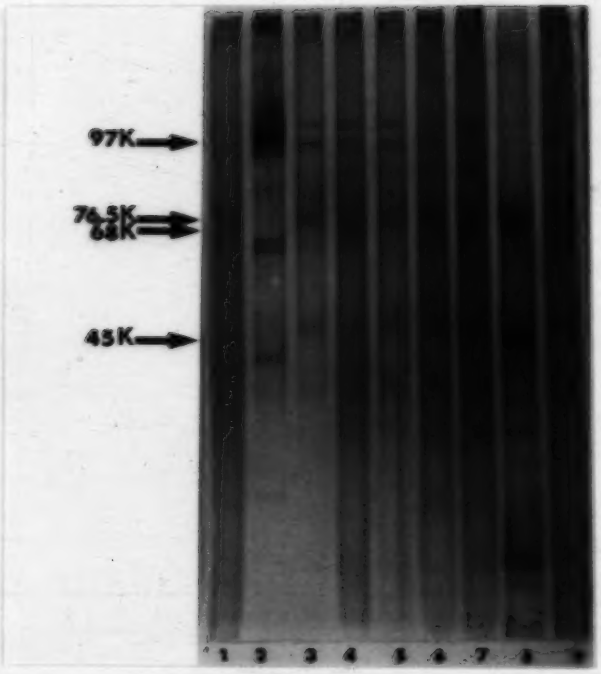
STEP 7. Retain fractions containing pure hemagglutinin for tryptic digest and HPLC analysis.

the fractions from the interface of the sucrose and detergent layers (Figure 1, step 2). This activity corresponded to the presence of hemagglutinin protein seen in lanes 6, 7 and 9 of Figure 2b.

Following the sucrose-tartrate gradient centrifugation, the detergent layer of the gradient containing the hemagglutinin protein was passed through an anion exchange column (Figure 1, step 3). The detergent eluted from the column during the wash cycle prior to starting the salt gradient. Fractions containing hemagglutinin protein eluted from the column at a salt concentration of 0.11 M and continued to do so until approximately 0.20 M NaCl. Hemagglutinin activity peaked one to three fractions ahead of the ultra-violet (UV) absorbance peak (Figure 3a). Those fractions containing hemagglutinin protein, as determined by SDS-PAGE analysis (Figure 3b), and hemagglutinin assay, were retained for further purification. At this point, the material pelleted at step 1 of the purification procedure was solubilized in Triton X-100 and analyzed by anion exchange chromatography (Figure 1, Step 1). This was done in an effort to recover any hemagglutinin which may have complexed with the insoluble material of the pellet. As seen in Figure 4a and 4b, substantial amounts of hemagglutinin were present. Those fractions containing hemagglutinin protein were pooled with those from the anion exchange column of figure 1, step 3.

Figure 2a. Ultra-violet absorbance of MVH hemagglutinin on sucrose-tartrate gradient. The bottom of the gradient is to the left. UV absorbance is (△—△), hemagglutinin activity is (▲—▲).

Figure 2b. Silver-stained SDS-PAGE analysis of MVH hemagglutinin on sucrose-tartrate gradient. Lane 2, molecular weight marker; 97,000 (phosphorylase b). Lane 8, molecular weight markers; 68,000 (BSA), 45,000 (ovalbumin). Lanes 1, 3-7 and 9 represent fractions 4-5, 6-7, 8-9, 10-11, 12-13, 14-15 and 16-17 respectively, of the above absorbance profile.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68K
45K
1 2 3 4 5 6 7 8 9

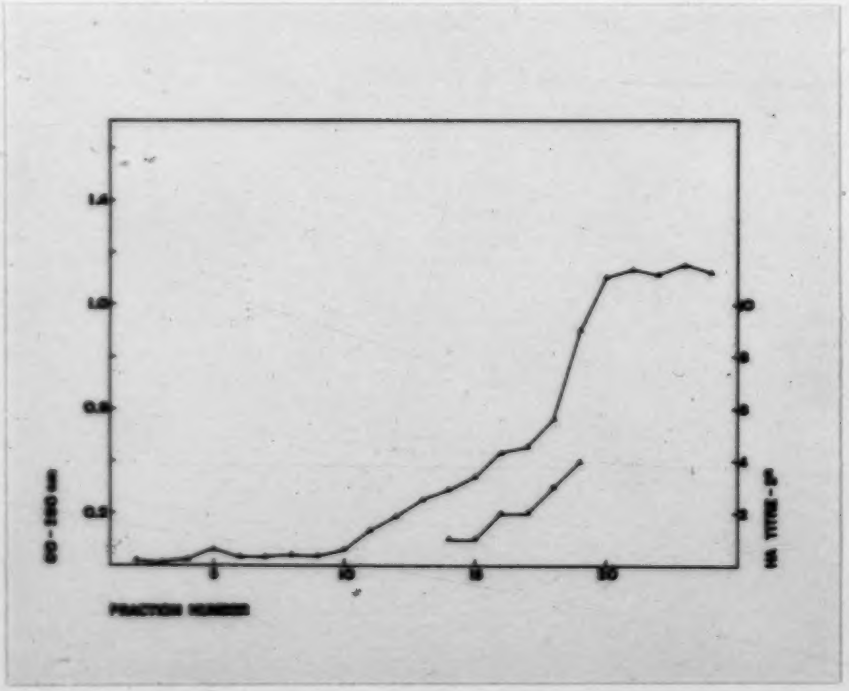
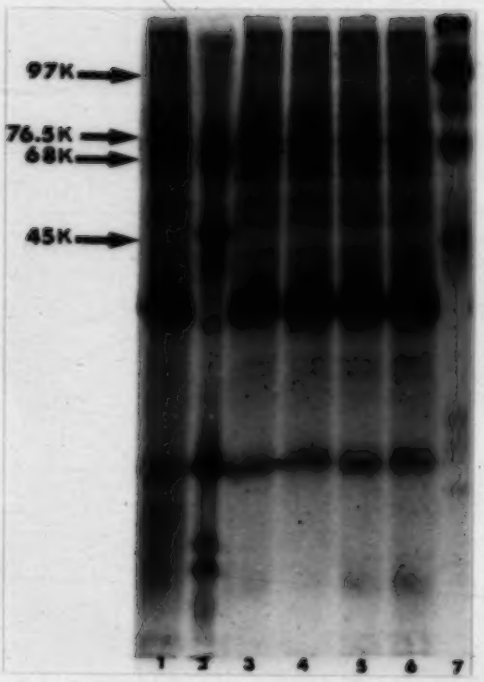

1.4
1.0
0.6
0.2
10
8
6
4
2
FRACTION NUMBER

Figure 3a. Anion exchange chromatographic profile (UV absorbance 280nm) of MVH hemagglutinin at step 3 of purification procedure. UV absorbance is (△—△), hemagglutinin activity is (▲—▲).

Figure 3b. Silver-stained SDS-PAGE analysis of MVH hemagglutinin from the above anion-exchange profile. Lanes 1 and 3-6 represent fractions 44-48 on the above profile. Lane 2, molecular weight markers; 68,000 (BSA) and 45,000 (ovalbumin). Lane 7, molecular weight marker; 97,000 (phosphorylase b).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45K
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2
3
4
5
6
7

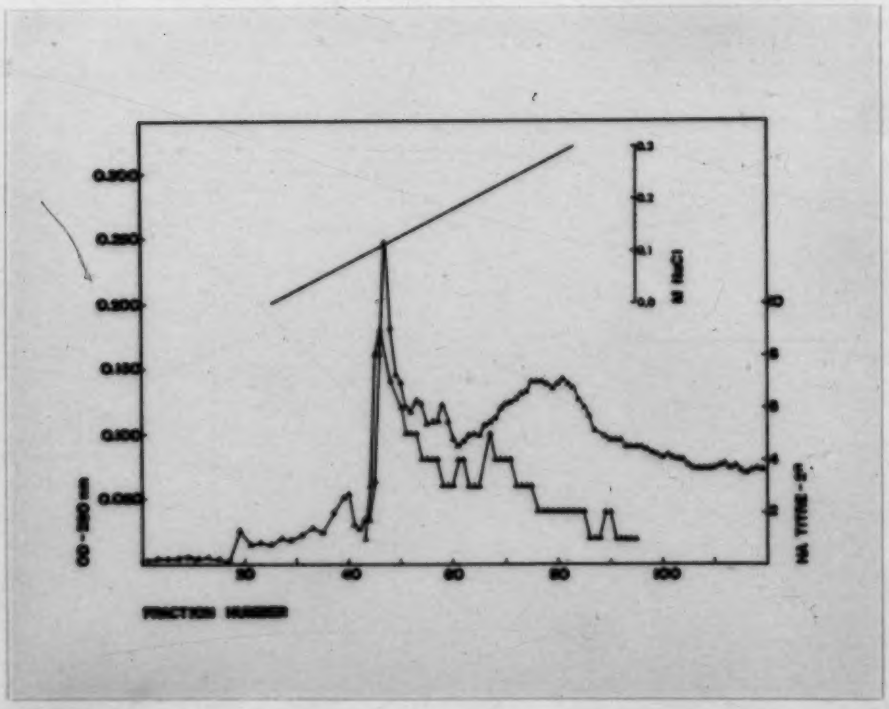
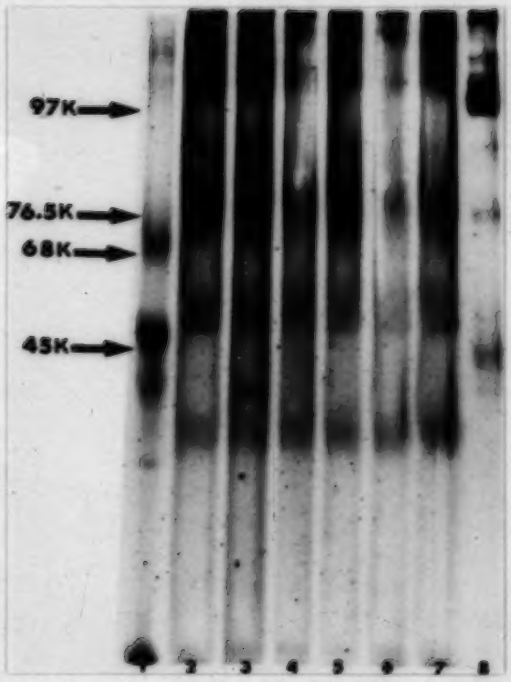

FRACTION NUMBER

Figure 4a. Anion-exchange chromatographic profile (UV absorbance 280nm) on the retreated Hallé pellet at step 2 in the purification procedure. UV absorbance is (△—△), hemagglutinin activity is (▲—▲).

Figure 4b. Silver-stained SDS-PAGE analysis of MVH hemagglutinin fractions collected in the above profile. Lane 1, molecular weight markers; 68,000 (BSA), 45,000 (ovalbumin). Lane 8, molecular weight marker; 97,000 (phosphorylase b). Lanes 2-7 represent fractions 44-49 on the above profile.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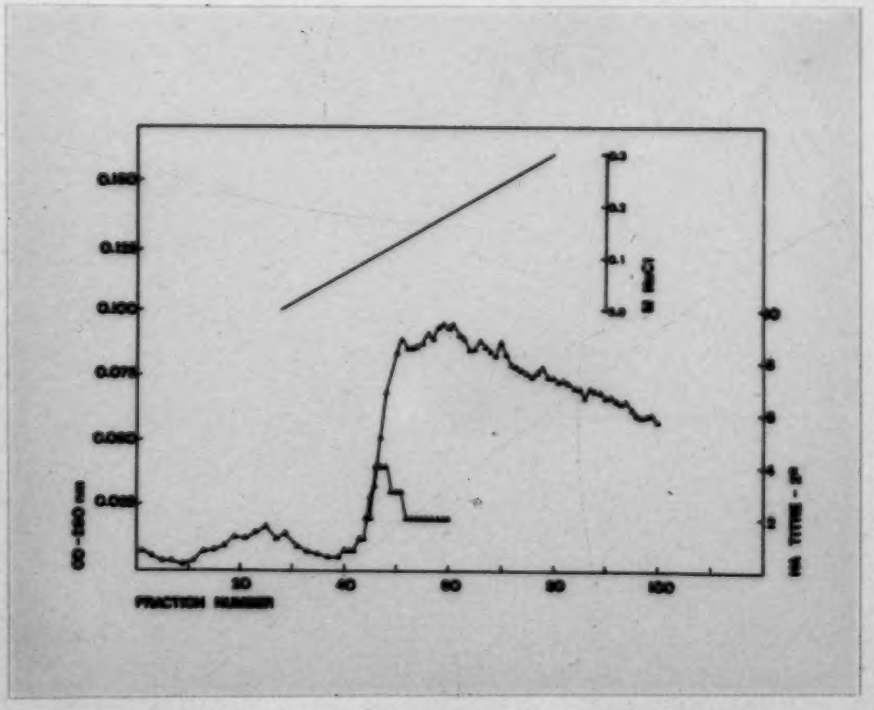
76.5K
68K
45K
1
2
3
4
5
6
7
8


FRACTION NUMBER

Addition of Triton X-100 to the hemagglutinin containing fractions, prior to centrifugation on the sucrose gradient, was done to solubilize any membrane complexes still containing hemagglutinin protein (Figure 1, Step 4). Centrifugation on the sucrose gradient concentrated a portion of the active hemagglutinin protein at the interface of the sucrose and detergent layers (Figure 5). The detergent layer of the gradient, was subjected to anion exchange chromatography (Figure 1, step 5; Figure 6a). Step 5). The purity of the hemagglutinin in this portion of the gradient was found to be greatly enchanced (Figure 6b). The purified hemagglutinin seen in Figure 7b and 8 was obtained from the hemagglutinin-containing fractions of the sucrose gradient. This was accomplished by repeating the addition of Triton X-100 to the hemagglutinin containing fractions and passage of this material through the anion exchange column (Figure 1, Step 6; Figure 7a). Those fractions still containing impurities were further treated with Triton X-100 and chromatographed on an anion exchange column.

During the development of this purification procedure, a "doublet" form of the hemagglutinin polypeptide was observed in a number of strains of measles virus (Edmonston strain; Figure 8). The doublets behaved very much like the single form of the polypeptide with the exception of the band position on SDS-PAGE analysis. It was noted that as the purity of the hemagglutinin preparation increased, the salt molarity at which either of the hemagglutinin forms eluted

Figure 5. UV absorbance profile (280nm) of MVH hemagglutinin fractions from sucrose gradient at step 4 of the purification procedure. The bottom of the gradient is to the left. UV absorbance is (△—△), hemagglutinin activity is (▲—▲).

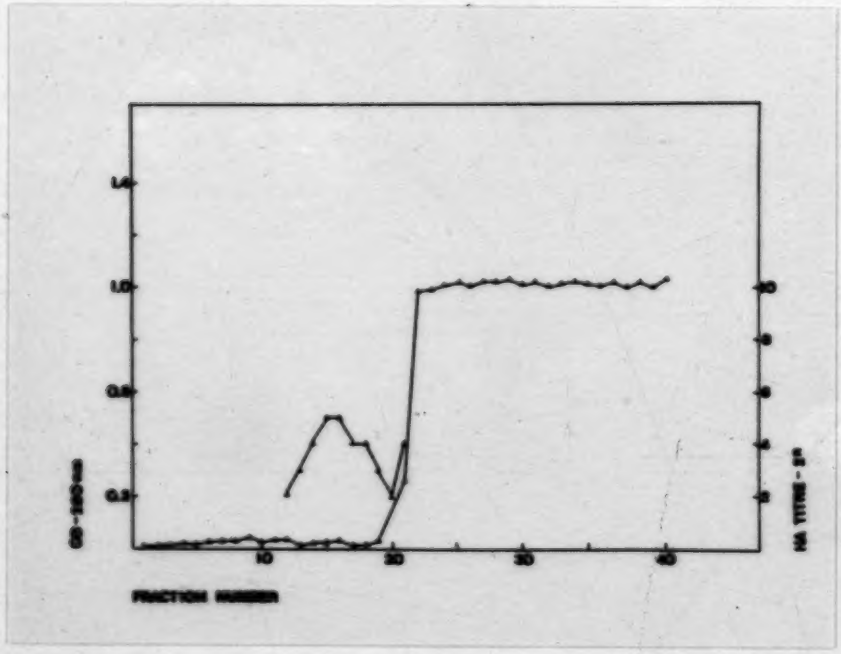
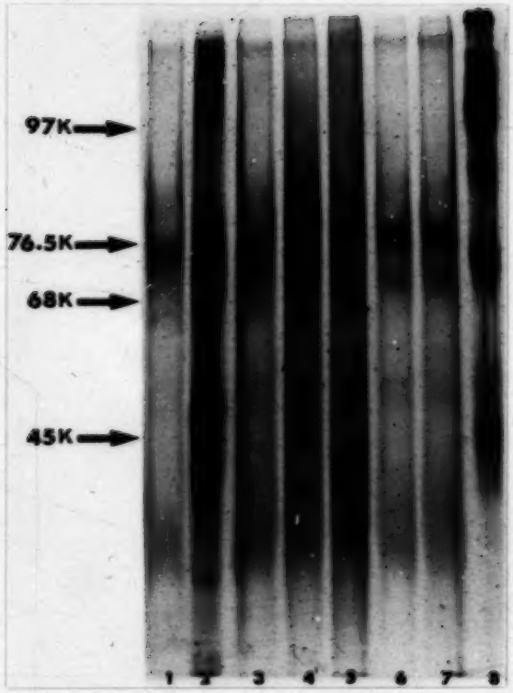

1.4
1.0
0.6
0.2
10
20
30
40
FRACTION NUMBER

Figure 6a. Anion-exchange chromatographic profile (UV absorbance 280nm) of MVH hemagglutinin contained in the detergent portion of the sucrose gradient (step 5 of the purification procedure). UV absorbance is (△—△), hemagglutinin activity is (▲—▲).

Figure 6b. Silver-stained SDS-PAGE analysis of MVH hemagglutinin fractions collected from the above gradient. Lane 2, molecular weight markers; 68,000 (BSA), 45,000 (ovalbumin). Lane 8, molecular weight marker; 97,000 (phosphorylase b). Lane 1 and 3-7 represent fractions 25-30 on the above profile.


97K
76.5K
68K
45K
1
2
3
4
5
6
7
8

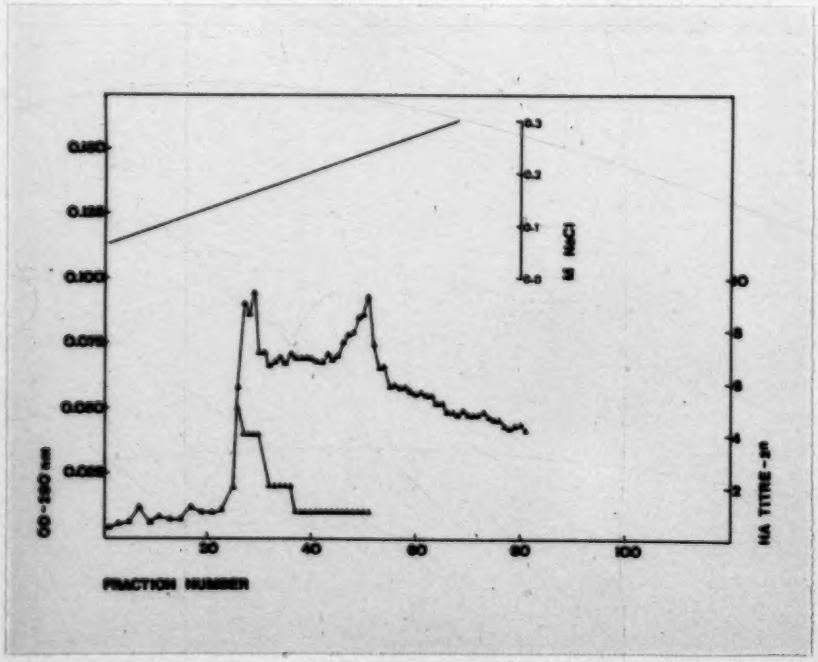
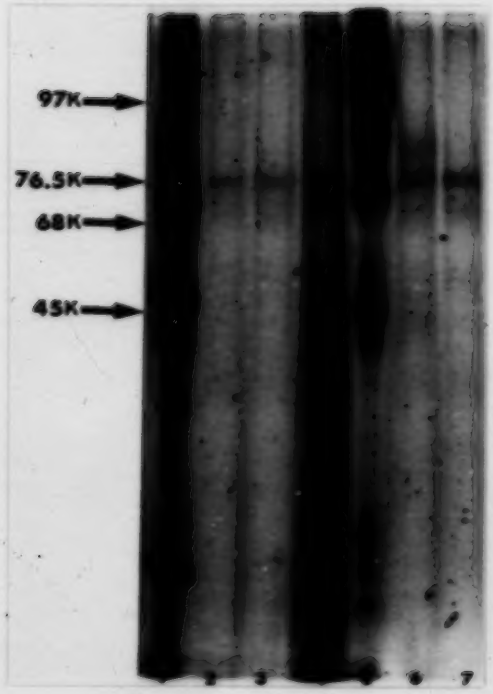

M NaCl
OD-280 nm
HA TITRE - μg
FRACTION NUMBER
20
40
60
80
100

Figure 7a. Anion-exchange chromatographic profile of purified MVH hemagglutinin, step 6 of the purification procedure. UV absorbance is (△—△), hemagglutinin activity is (▲—▲).

Figure 7b. Silver-stained SDS-PAGE analysis of purified MVH hemagglutinin collected from the above profile. Lane 1, molecular weight markers; 68,000 (BSA), 45,000 (ovalbumin). Lane 5, molecular weight marker; 97,000 (phosphorylase b). Lanes 2-4, 6 and 7 represent fractiions 43-47 respectively of the above profile. Lane 2 contains approximately 120 ng of hemagglutinin.


97K
76.5K
68K
45K
5
6
7

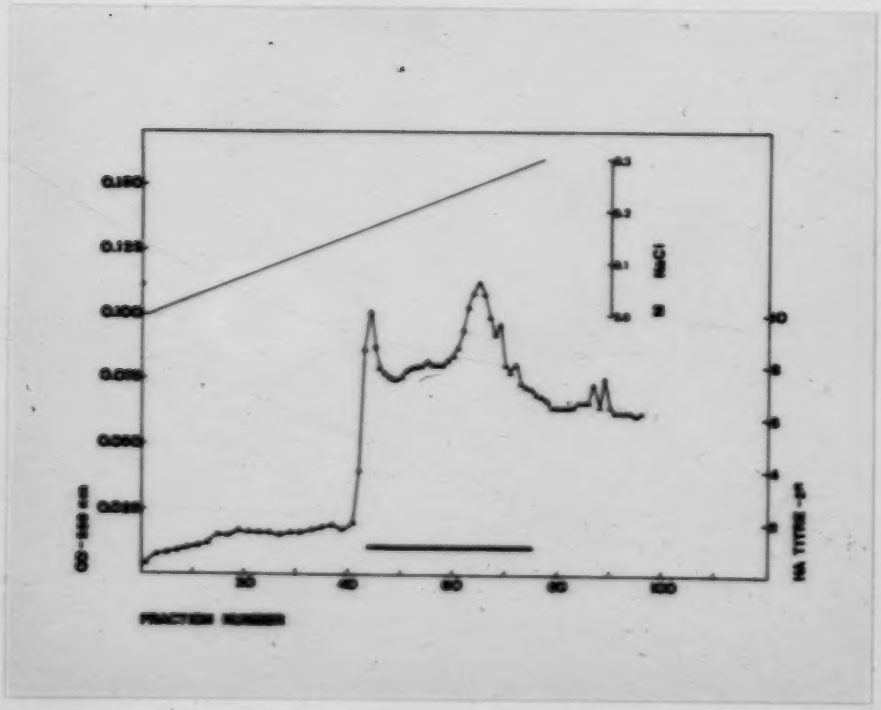
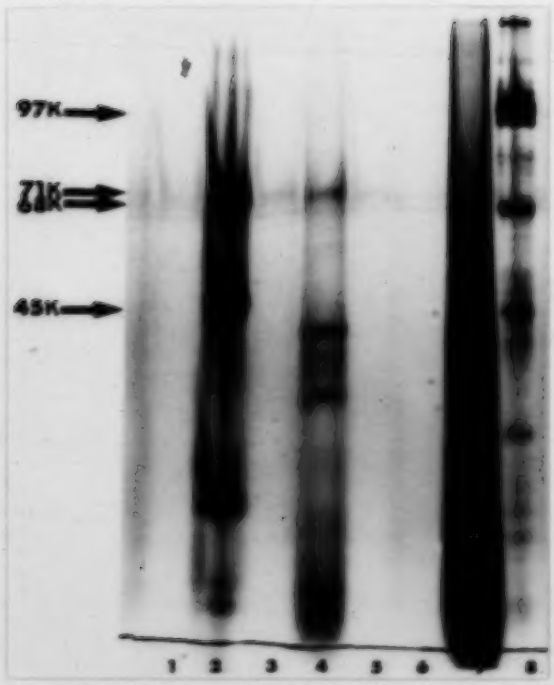

FRACTION NUMBER
NaCl
M

Figure 8. Silver-stained SDS-PAGE analysis of purified MVE hemagglutinin doublets. Lane 2, molecular weight markers; 68,000 (BSA), 45,000 (ovalbumin). Lane 8, molecular weight marker; 97,000 (phosphorylase b). Molecular weight of the hemagglutinin doublets is approximately 71,000 and 66,000 (lanes 1, 3 and 4).


97K
68K
43K
1
2
3
4
5
6
8

from the anion exchange column also increased. The hemagglutinin polypeptides shown in Figure 7b and 8 eluted at 0.17 M NaCl as opposed to 0.11 M NaCl shown in Figure 3a. The hemagglutinin activity of the Hallé doublets was found to be comparable to that of the single polypeptide conformation whenever the doublets were present.

C. Assays

a. Hemagglutinin Assays

The initial hemagglutination titre of the purified Edmonston strain was 1:32. Hemagglutinin activity was not detected at any other step during purification. The purified Hallé strain had an initial hemagglutination titre of 1:16. After passage through the first anion exchange column (Figure 3a), the titre increased to 1:128. As purity of the preparation increased, the hemagglutinin activity gradually decreased to the minimal level seen in Figure 7b.

b. Protein Assays

The initial protein yield from the purified Edmonston strain averaged 5.0 ug/10^6 cells. The total amount of hemagglutinin recovered averaged 0.086 ug/10^6 cells which was equivalent to 1.70% of the initial amount of protein. The initial protein content of the purified Hallé strain averaged 7.0 ug/10^6 cells. The final amount of hemagglutinin recovered averaged 0.165 ug/10^6 cells or 2.40% of the

initial amount of protein.

D. Iodination of Purified Hemagglutinin

Attempts to radio-iodinate the purified Hallé hemagglutinin were done as an alternative means of confirming its purity (data not shown). Difficulty was encountered in preventing the carrier BSA protein used in this procedure from acquiring any of the added radiolabel.

E. HPLC Analysis of Hemagglutinin Tryptic Digests

A trypsin self-digest control was done for both the Edmonston (Figure 9b) and the Hallé (Figure 10a) hemagglutinin tryptic digests. The amount of trypsin used in each control run was equal to that used in the corresponding hemagglutinin digest. In this way, trypsin peptides generated as a result of autodigestion could be recognized. The peptide maps of the hemagglutinin and tryptic digests were plotted at the same attenuation value to facilitate, as accurately as possible, a comparison of the relative heights and retention times of each peak detected.

The Hallé hemagglutinin peptide profile was generated by digesting 20 ug of hemagglutinin. The Edmonston peptide profile was generated by digesting 16 ug of Edmonston hemagglutinin. Significant peptide peaks were noted throughout the peptide profile of each strain, (Figure 9a and 10b). Figure 11 provides a visual comparison of the Hallé and Edmonston hemagglutinin tryptic profiles. The

majority of the peaks in both profiles elute at similar times however, some differences can be noted. A decrease in UV absorbance seen at 25 min of the Edmonston hemagglutinin digest corresponds to an increase in UV absorbance at the same time in the Hallé digest. A significant difference between the two profiles is the presence of a group of three peptide peaks seen in the Hallé profile at 35-42 min (38%-42% acetonitrile), but which are absent from the Edmonston hemagglutinin tryptic profile.

Figure 9a. HPLC tryptic profile of Edmonston hemagglutinin peptides.

Figure 9b. HPLC tryptic profile of trypsin autodigest control.

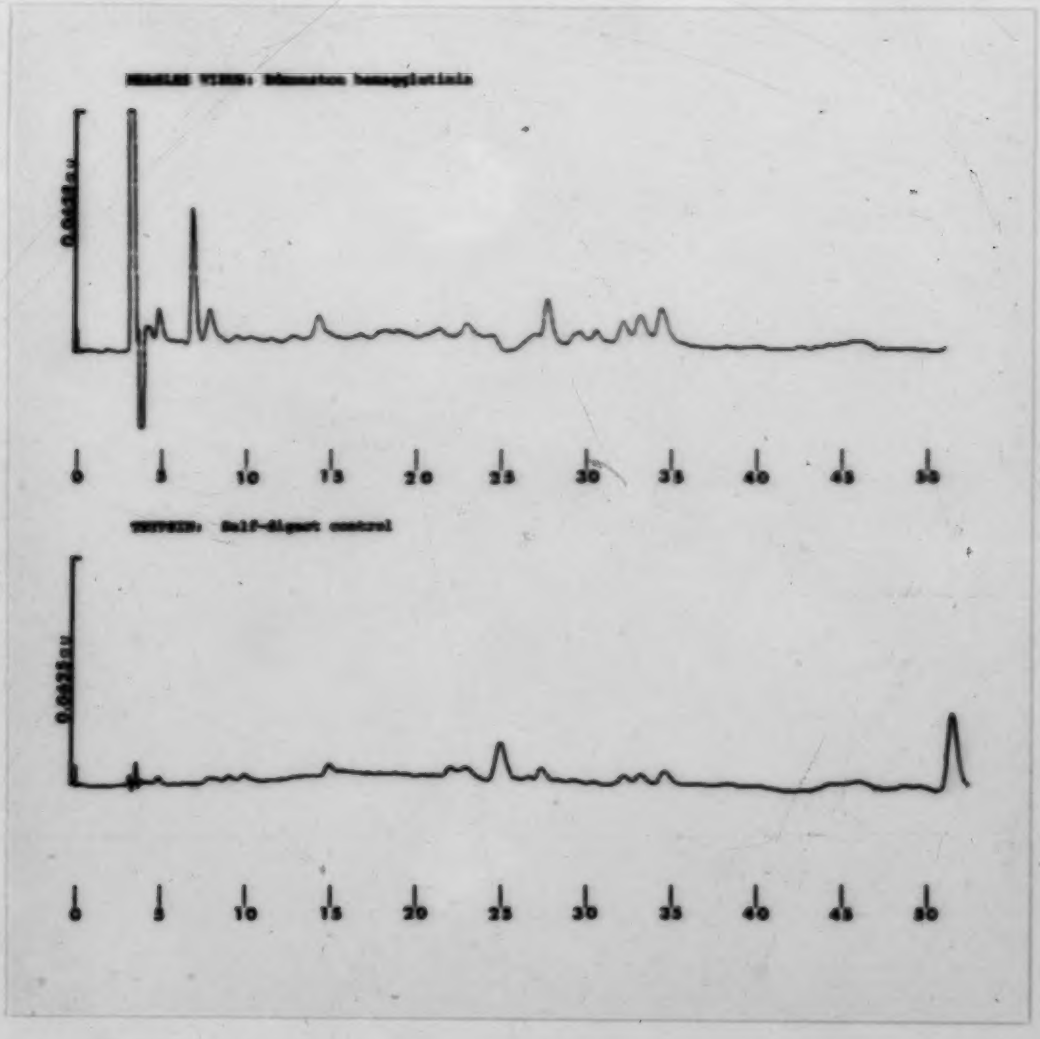

0 5 10 15 20 25 30 35 40 45 50
0 5 10 15 20 25 30 35 40 45 50

Figure 10a. HPLC tryptic profile of trypsin autodigest control.

Figure 10b. HPLC tryptic profile of Hallé hemagglutinin peptides.

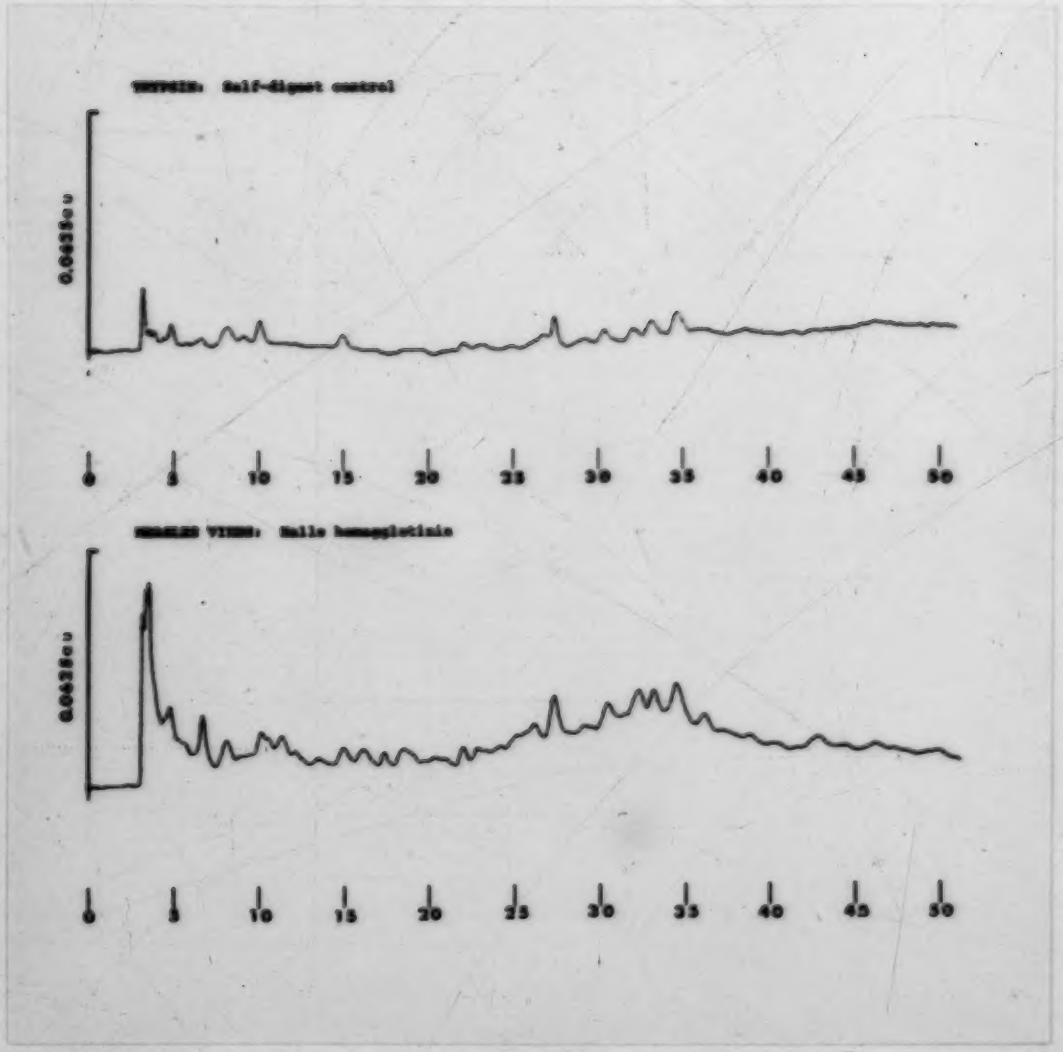
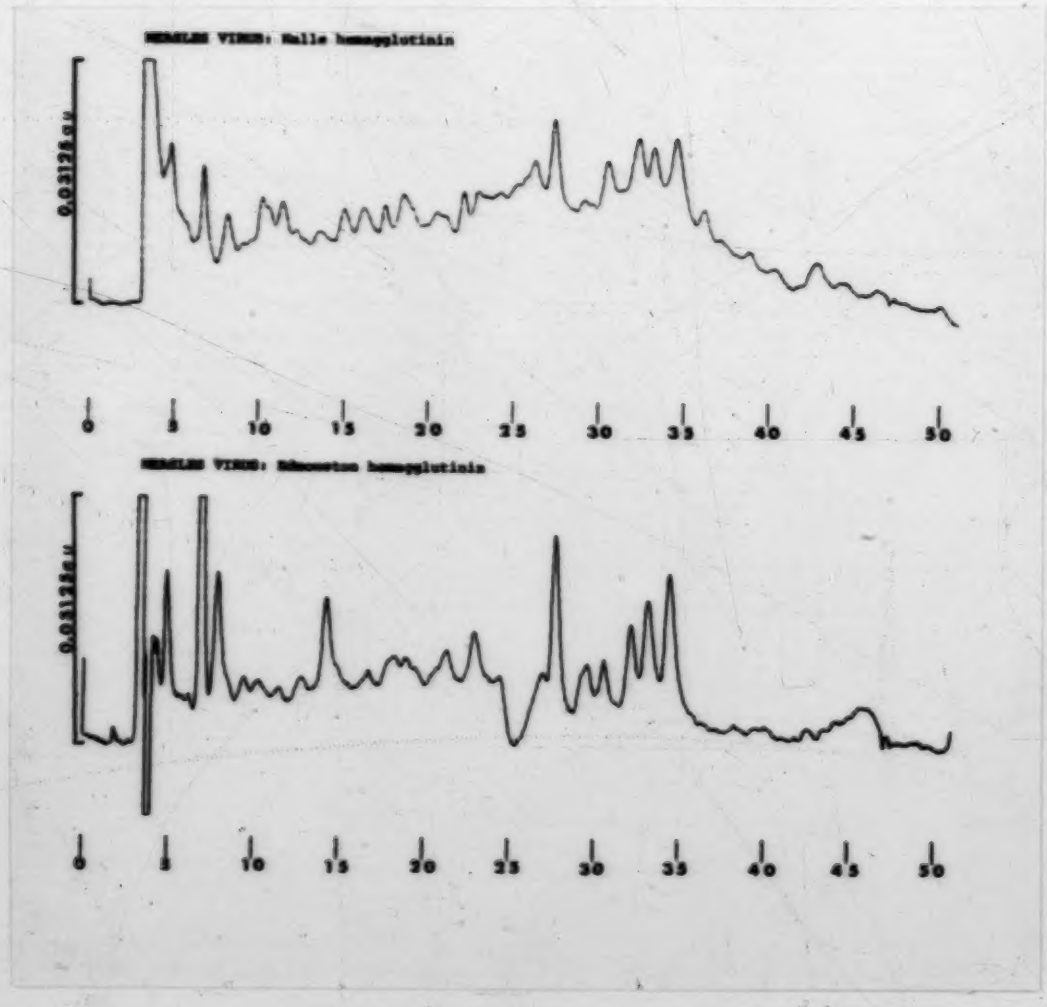

0.0438 o.u
0 5 10 15 20 25 30 35 40 45 50
0.0438 o.u
0 5 10 15 20 25 30 35 40 45 50

Figure 11. HPLC tryptic profiles of the Edmonston and Hallé hemagglutinin peptides.


0.03128 a.u.
0
5
10
15
20
25
30
35
40
45
50
0.03128 a.u.
0
5
10
15
20
25
30
35
40
45
50

IV. Discussion

The procedure developed in this study for the purification of measles virus hemagglutinin encountered difficulties which numerous other researchers have referred to with respect to this virus. The close association between viral and cellular components within the virion membrane (Mountcastle and Choppin, 1977) and the lack of inhibition of cellular biosynthesis during measles virus replication (Bellini et al., 1983; Rima, 1983) are likely responsible for these problems. The goal of this project was to develop a simple purification method to obtain biologically active hemagglutinin so that comparative analysis of hemagglutinin tryptic digests by high performance liquid chromatography could be done.

The hemagglutinin, being a trans-membrane protein, is embedded in the cellularly-derived virion membrane. Treatment of the membrane with a non-ionic detergent causes disruption of the membrane by solubilizing its components. This action releases the hemagglutinin from the membrane. The anion exchange column used following the detergent treatment separated the freed hemagglutinin peplomers from other membrane-associated components. Although elution of the hemagglutinin polypeptide from SDS-PAGE gel slices would provide a more rapid source of purified hemagglutinin, the structure and biological activity of the protein would be lost. Affinity chromatography, using immobilized antibodies directed against the hemagglutinin being studied, was

considered as a method of purification. However, monoclonal antibodies available to this project had no affinity for the Hallé hemagglutinin. In this case, anion exchange chromatography gave the best separation results.

In each of the anion exchange chromatographs, two significant UV absorbance peaks were noted. The first of these peaks being very sharp and abrupt. The fractions collected at this point contained active hemagglutinin protein as determined by hemagglutination assay and SDS-PAGE analysis. Fractions collected from the second peak contained lesser amounts of hemagglutinin in association with large amounts of impurities, the majority of which consisted of 200,000 and 60,000 molecular weight components.

The hemagglutination titre of the Hallé hemagglutinin, following the initial anion exchange column, (Figure 3), increased three-fold with respect to the whole virus preparation. This increase in titre would indicate that accessibility to and/or activity of the hemagglutinin in the intact membrane is inhibited by other components of the virion membrane. Detergent treatment and passage through an anion exchange column significantly reduces this inhibition. As purification of the Hallé hemagglutinin continued, the relative hemagglutination titre decreased. Hydrophobic portions of the hemagglutinin, which, *in situ*, span the virion membrane, may promote aggregation of the hemagglutinin peplomers once they are free of the membrane. Characteristics of the carbohydrate group bound to the

protein will also influence the degree of aggregation. Aggregates of this protein in its purified form have been observed in the absence of a detergent (Varsanyi, Utter and Norrby, 1983). These structures, if present, could significantly decrease the hemagglutination activity of the preparation due to steric hinderance. Hemagglutinin activity in the Edmonston preparation was observed only with the purified whole virus. Absence of hemagglutinin activity throughout the remainder of the purification procedure implies there is either a difference between the Hallé and Edmonston hemagglutinin structures or that activity of the Edmonston hemagglutinin is dependent on an intact virion membrane as opposed to membrane fragments. Studies on the structure and function of the viral membrane must be done before any conclusions may be reached on this difference.

During the development of this purification procedure, SDS-PAGE analysis revealed the presence of a doublet as well as the usually observed single band of the hemagglutinin polypeptide. This observation was made with the Edmonston and Hallé strains, as well as with other measles virus strains (L. Chui and R. Marusyk, personal communication; Stephenson et al., 1981). Bellini et al., (1983), have shown that tunicamycin-induced inhibition of glycosylation of the hemagglutinin of the Mantooth strain resulted in the production of hemagglutinin polypeptide precursors. These precursors ranged in molecular weight from 65,000 for the unglycosylated precursor to 76,000 for the fully

glycosylated hemagglutinin. The doublets observed in this study could represent precursors of the hemagglutinin protein. Further studies on the structure of these polypeptides are necessary in order to determine their relatedness to the hemagglutinin protein.

The molecular weight of the measles hemagglutinin polypeptide seems to be dependent on both the strain of measles studied and the technique used to isolate the protein. The molecular weight of the hemagglutinin from both the Mantooth and Edmonston strains has been calculated to be approximately 76,000 (Bellini, Trudgett McFarlin, 1979; Bellini et al., 1981). Graves et al., (1978), has determined the molecular weight of the Edmonston hemagglutinin to be 80,000. In this project, the molecular weight of the Hallé hemagglutinin was approximately 77,000 and that of the Edmonston was approximately 77,000. The molecular weight of the doublet seen in association with the Edmonston strain (Figure 8) was approximately 66,000 and 71,000 for the respective bands.

Recovery of hemagglutinin protein from the purified viral material was low. A major contributing factor is the inability of the detergent to solubilize the virion membrane efficiently. Other non-ionic detergents such as octyl-β-D-glucopyranoside, Nonidet P-40 and Tween-20 did not improve the yield of hemagglutinin. A second factor was the loss of hemagglutinin protein which complexed with insoluble components of the virion in the pelleted material (Figure 1,

step 1). Partial recovery of the lost hemagglutinin was accomplished by treating the pellet with Triton X-100 and analyzing the product by anion exchange chromatography. Improvements in the efficiency of solubilizing the viral membrane would significantly increase the yield of hemagglutinin.

The purity of the hemagglutinin preparation in this project was determined by the presence and/or absence of contaminating polypeptide bands observed in silver-stained SDS-PAGE gels. The silver staining procedure used here was a modification of the method proposed by Wray et al., (1981). This method is capable of detecting nanogram quantities of proteins in SDS-PAGE gels. This agrees with the result seen in Figure 7b, lane 2, in which 120 ng of pure hemagglutinin were quite easily detected.

To confirm the purity of the Hallé hemagglutinin preparation, iodination of the purified hemagglutinin was done. However, the bands visualized on the autoradiogram correspond to the BSA carrier protein used in this experiment (data not shown). Attempts to correct this problem were unsuccessful.

Reverse-phase high performance liquid chromatography was the method of choice for analysis of the tryptic peptides. Ion-exchange high performance liquid chromatography was not used since charges on the peptides present would be predominantly polar rather than ionic. Gel exclusion high performance liquid chromatography would not

provide a separation of peptides which could adequately be used to determine any differences between the two hemagglutinin proteins being studied. Two dimensional autoradiography was not considered due to its lack of speed, its poor resolution and its incompatibilty to computer interpretation.

The hemagglutinin tryptic profiles produced in this study were not expected to be dramatically different since the hemagglutinin proteins were both derived from measles virus - a supposedly invariant virus (Marusyk, 1983). However, as observed in this study, a group of three peptide peaks were detected in the latter portion of the Hallé tryptic profile, but were absent in the Edmonston profile. The successful detection and resolution of this difference within a relatively short analysis time substantiate the reasons for the use of a high performance liquid chromatography system as the analytical tool for this study.

Each of the digests done was influenced by such factors as: i) the ability of the trypsin to maintain a consistant level of activity during the digestion period and; ii) by the accessibility of arginine and lysine cleavage sites on the hemagglutinin protein with respect to the position of the carbohydrate component of the protein. In order to minimize the influence of the first variable, both digests were done using the same protein:enzyme (w:w) ratio. As well, the digests were done simultaneously to ensure that any temperature and time influences would be common to both.

The second variable is one in which the influence is not yet known. Studies such as these will help to more accurately define the properties of this protein. The extra peptide peaks observed in the latter portion of Hallé hemagglutinin tryptic profile, indicate the existance of a number of more hydrophobic sequences of amino acids in this hemagglutinin as opposed to the Edmonston hemagglutnin. Amino acid analysis and structure-function relationship studies will be required before the significance of these extra peptides with respect to the active site(s) of the Hallé hemagglutinin protein can be determined.

A final step in this comparative study would be to subject these profiles to computer analysis. Time restraints and computer incompatibility problems prevented this from being done in this study. Nevertheless, with the aid of such an analysis, it is hoped that additional clues which differentiate the various measles virus isolates from one another can be found. The importance of this differentiation is apparent when considering the ability of a given strain of virus to infect a specialized tissue which is not susceptibile to infection by other strains of the same virus. Such is the case of SSPE linked measles virus strains, ie. the Hallé strain, which are capable of infecting central nervous system tissue whereas other strains are not, ie. the Edmonston strain. The relevance of the findings of this study will become evident as more knowledge about other latent, central nervous system

diseases of viral etiology is acquired.

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